In Vitro Susceptibility of *Malassezia pachydermatis* Isolates from Canine Skin with Atopic Dermatitis to Ketoconazole and Itraconazole in East Asia

Shion WATANABE[1], Anna KOIKE[1], Rui KANO[1]*, Masahiko NAGATA[2], Charles CHEN[3], Cheol-Yong HWANG[4], Atsuhiko HASEGAWA[5] and Hiroshi KAMATA[1]

[1]*Department of Pathobiology, Nihon University School of Veterinary Medicine, 1866 Kameino, Fujisawa, Kanagawa 252–0880, Japan*
[2]*ASC Dermatology Service, 1–3–2 Higashicho, Jindaiji, Chofu, Tokyo 182–0012, Japan*
[3]*Asian Veterinary Specialist Referral Center, 1st Floor, No. 109, Section 4, XinYi Road, Taipei 106, Taiwan*
[4]*Department of Internal Medicine (Dermatology), College of Veterinary Medicine, Seoul National University, Seoul 151–742, Korea*
[5]*Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192–0395, Japan*

ABSTRACT. Topical or oral azole antifungals are commonly used in canine atopic dermatitis (AD), as the lipophilic yeast *Malassezia pachydermatis* exacerbates canine AD. To examine whether canine AD lesions harbor azole-resistant *M. pachydermatis* isolates in East Asia, we investigated the *in vitro* susceptibility of *M. pachydermatis* isolates to ketoconazole (KTZ) and itraconazole (ITZ) obtained from AD lesions of canines in Japan, Korea and Taiwan. The minimum inhibitory concentrations (MICs) of KTZ and ITZ were measured by the E-test using Sabouraud dextrose agar with 0.5% Tween 40. The MICs of KTZ and ITZ for isolates from canines with AD were significantly higher than the MICs for isolates from healthy canines. Our findings suggested that the clinical isolates from canine AD skin lesions were less susceptible to azoles than those from normal canine skin in East Asia.
KEY WORDS: azole, drug susceptibility, E-test, *Malassezia pachydermatis*.

Canine atopic dermatitis (AD) is frequently treated with topical or oral azole antifungals, as the lipophilic yeast *Malassezia pachydermatis* is recognized as an exacerbating factor of canine AD [6, 8]. Previously, we reported that a clinical isolate of *M. pachydermatis* was resistant to itraconazole [9]. Cafarchia *et al.* in Italy reported that clinical isolates of *M. pachydermatis* from canine skin lesions had low susceptibility to azoles compared with those from normal canine skin [1]. We are anxious about the phenomenon worldwide, because we frequently use azoles in shampoo therapy for canine AD. To confirm these observations, we investigated the susceptibility of *M. pachydermatis* isolates obtained from canine AD skin lesions in East Asia to azoles. Specifically, to test the hypothesis that canine AD lesions harbor azole-resistant *M. pachydermatis* isolates, the *in vitro* susceptibility of *M. pachydermatis* isolates from AD skin lesions of canines in Japan, Taiwan and Korea was examined in a two-year period between 2010 and 2011. This is the first epidemiological investigation for *in vitro* susceptibility of *M. pachydermatis* in East Asia.

Seventy-two isolates of *M. pachydermatis* were obtained from 24 healthy dogs (three isolates from each dog in Japan) with no skin disease. On the other hand, 110 isolates were obtained from 42 dogs (25 isolates from 11 dogs in Japan, 22 isolates from 11 dogs in Korea and 63 isolates from 20 dogs in Taiwan) with AD lesions. The diagnosis of atopic dermatitis was made according to the criteria described by Miller *et al.* and Olivry *et al.* [6, 10], and all affected dogs had typical lesions with greasy or waxy exudates. Each sample was collected from at least two to three areas of the external ear, paw and abdomen and cultured on CHROMagar *Malassezia* medium (CHROMagar, Paris, France) [4]. Morphological examination and molecular identification of *M. pachydermatis* were performed as described by previous reports [3, 5].

Stock inoculum suspensions were prepared from 7-day-old cultures grown on modified Dixon's agar (3.6% malt extract, 0.6% peptone, 2.0% desiccated ox bile, 1.0% Tween 40, 0.2% glycerol, 0.2% oleic acid and 1.2% agar, pH 6.0) [3] at 32°C. The final concentration of the stock inoculum suspensions was adjusted spectrophotometrically to the optical density of 1.0 (OD 530 nm) in sterile 0.9% saline.

The *in vitro* activity of KTZ and ITZ against clinical isolates of *M. pachydermatis* was investigated by the E-test. E-test gradient strips for ITZ and KTZ were obtained from AB BIODISK (Solna, Sweden). The E-test was performed according to the manufacturer's instructions using 20 m*l* Sabouraud dextrose agar (1% peptone, 2% dextrose and 2% agar) with 0.5% of Tween 40 in 90-mm Petri plates. Minimum inhibitory concentrations (MICs) were determined after a 72-hr incubation at 32°C. The E-test MIC was determined three times for each isolate. For quality control, the strain *Candida parapsilosis* ATCC 22019 strain was used in each experiment to check the accuracy of drug dilution (Customer Information Sheet CIS005 AB BIODISK 2004-01, AB BIODISK).

The E-test MIC was deemed to be the lowest drug concen-

Table 1.   Ketoconazole MICs (μg/m*l*) for isolates of *M. pachydermatis*

	Healthy dogs, Japan	AD, Japan	AD, Korea	AD, Taiwan
Range	0.019–0.125	0.016–21	0.058–3	0.048–8
MIC_{50}	0.094	0.38	0.33	1.3
MIC_{90}	0.125	5.2	1.6	4.21
MICm (SD)	0.09 (0.036)	1.35 (2.45)	0.722 (1.03)	1.9 (1.77)
SSD		$P<0.05$	$P<0.05$	$P<0.05$

MIC mean (MICm) and standard deviation (SD) values are also shown. Statistically significant difference (SSD) indicates that the normal group was compared with each AD group using the Sidak multiple comparison procedure.

Table 2.   Itraconazole MICs (μg/m*l*) for isolates of *M. pachydermatis*

	Healthy dogs, Japan	AD, Japan	AD, Korea	AD, Taiwan
Range	0.012–0.125	0.032–16	0.034–15	0.031–21
MIC_{50}	0.047	0.5	0.27	1.6
MIC_{90}	0.119	3	7.09	5.36
MICm (SD)	0.059 (0.033)	3.12(4.46)	2.53(5.53)	2.75(3.94)
SSD		$P<0.05$	$P<0.05$	$P<0.05$

MIC mean (MICm) and standard deviation (SD) values are also shown. Statistically significant difference (SSD) indicates that the normal group was compared with each AD group using the Sidak multiple comparison procedure.

tration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. Each isolate was tested three times on separate occasions. The MICs of the two drugs for isolates from normal and AD skin were compared using the Sidak multiple comparison procedure (Microsoft Excel 2010, Tokyo, Japan).

The MICs of KTZ and ITZ for the 72 isolates of *M. pachydermatis* from healthy dogs and 110 isolates from AD-afflicted dogs are shown in Tables 1 and 2. The MICs of KTZ and ITZ for isolates from canines with AD were significantly higher than the MICs for isolates from healthy canines ($P<0.05$).

Differences in the MICs of two drugs for isolates from each AD group were assessed with the Sidak multiple comparison procedure (Microsoft Office Excel 2010). No significant differences in MICs were identified for isolates between the AD groups.

Our findings are similar to those of Cafarchia *et al*. [1], who observed that the clinical isolates from canine skin lesions were less susceptible to azoles than those from normal canine skin, suggesting that the phenomenon may also be seen in East Asia. This phenomenon might occur worldwide.

The MIC ranges for 50% inhibition (MIC_{50}) and 90% inhibition (MIC_{90}) of clinical isolates obtained by the modified CLSI M27-A2 test and the E-test were different (Tables 1 and 2) [1]. We suspected that the extent of resistance of Asian clinical isolates had progressed farther than that of Italian clinical isolates.

Unfortunately, the treatment history of each canine with AD from which the samples were obtained was not available. Due to the fact that *Malassezia* is considered to be an exacerbating factor of canine AD, we expect that many of the cases of canine AD were treated with either oral or topical azoles.

Resistance of fungi to azoles may be caused by such things as by alterations in sterol biosynthesis, by mutations in the drug target enzyme, sterol 14α-demethylase, which lowers its affinity for the azole, by increased expression of the *ERG11* gene encoding for this enzyme or by overexpression of genes coding for membrane transport proteins of the ABC transporter (CDR1/CDR2) or the major facilitator (MDR1) superfamilies [7]. These mutations and overexpression of genes can be advantaged by long time azole therapy. Therefore, low susceptibility of *M. pachydermatis* to azoles is also speculated to result from the mutation of *ERG11* or increasing over expression of the drug resistance genes. Future investigation on sequencing and expression of these genes is required to understand the drug resistance mechanisms of *M. pachydermatis*.

The E-test is readily available and is a relatively easy method of determining the susceptibility of yeasts and molds to antifungals [2]. However, the breakpoints (μg/m*l*) for antifungal drugs for the test were not available from the manufacturer. A future study is required to define the breakpoints (μg/m*l*) of KTZ and ITZ using the E-test for the treatment of *M. pachydermatis*-associated diseases and to examine the mechanism of drug resistance of this yeast.

REFERENCES

 1. Cafarchia, C., Figueredo, L. A., Iatta, R., Montagna, M. T. and Otranto, D. 2012. *In vitro* antifungal susceptibility of *Malasse-*

zia pachydermatis from dogs with and without skin lesions. *Vet. Microbiol.* **155**: 395–398. [Medline] [CrossRef]

2. Cantón, E., Espinel-Ingroff, A. and Pemán, J. 2009. Trends in antifungal susceptibility testing using CLSI reference and commercial methods. *Expert. Rev. Anti. Infect. Ther.* **7**: 107–119. [Medline] [CrossRef]

3. Guillot, J., Guého, E. and Chermette, R. 1995. Confirmation of the nomenclatural status of *Malassezia pachydermatis*. *Antonie van Leeuwenhoek.* **67**: 173–176. [Medline] [CrossRef]

4. Kaneko, T., Makimura, K., Abe, M., Abe, M., Shiota, R., Nakamura, Y., Kano, R., Hasegawa, A., Sugita, T., Shibuya, S., Watanabe, S., Yamaguchi, H., Abe, S. and Okamura, N. 2007. A revised culture based identification system for *Malassezia*. *J. Clin. Microbiol.* **45**: 3737–3742. [Medline] [CrossRef]

5. Kobayashi, T., Kano, R., Nagata, M., Hasegawa, A. and Kamata, H. 2011. Genotyping of *Malassezia pachydermatis* isolates from canine healthy skin and atopic dermatitis by internal spacer 1 (IGS1) region analysis. *Vet. Dermatol.* **22**: 401–405. [Medline] [CrossRef]

6. Miller, W. H. Jr., Griffin, C. F. and Campbell, K. L. 2012. *Malas-sezia* dermatitis and canine atopic dermatitis. pp. 243–249 and 365–388. *In*: Muller & Kirk's Small Animal Dermatology, 7th ed., ELSEVIER MOSBY, St. Louis.

7. Morschhäuser, J. 2002. The genetic basis of fluconazole resistance development in *Candida albicans. Biochim. Biophys. Acta* **1587**: 240–248. [Medline] [CrossRef]

8. Negre, A., Bensignor, E. and Guillot, J. 2009. Evidence-based veterinary dermatology: a systemic review of intervention for *Malassezia* dermatitis in dogs. *Vet. Dermatol.* **20**: 1–12. [Medline] [CrossRef]

9. Nijima, M., Kano, R., Nagata, M., Hasegawa, A. and Kamata, H. 2011. An azole-resistant isolate of *Malassezia* pachydermatis. *Vet. Microbiol.* **149**: 288–290. [Medline] [CrossRef]

10. Olivry, T., DeBoer, D. J., Favrot, C., Jackson, H. A., Mueller, R. S., Nuttall, T. and Prélaud, P. 2010. International Task Force on Canine Atopic Dermatitis. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. *Vet. Dermatol.* **21**: 233–248. [Medline] [CrossRef]